Endocannabinoid signaling via cannabinoid receptor 1 is involved in ethanol preference and its age-dependent decline in mice

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Cannabinoids and ethanol can activate the same reward pathways, which could suggest endocannabinoid involvement in the rewarding effects of ethanol. The high ethanol preference of young (6–10 weeks) C57BL/6J mice is reduced by the cannabinoid receptor 1 (CB1) antagonist SR141716A to levels observed in their CB1 knockout littermates or in old (26–48 weeks) wild-type mice, in both of which ethanol preference is unaffected by SR141716A. Similarly, SR141716A inhibits food intake in food-restricted young, but not old, wild-type mice. There are no age-dependent differences in the tissue levels of the endocannabinoids anandamide and 2-arachidonoylglycerol or the density of CB1 in the hypothalamus, limbic forebrain, amygdala, and cerebellum. CB1-stimulated guanosine 5′-[γ-thio]triphosphate (GTP[γS]) binding is selectively reduced in the limbic forebrain of old compared with young wild-type mice. There is no age-dependent difference in G_i or G_o subunit protein expression in the limbic forebrain, and the selective reduction in GTP[γS] labeling in tissue from old mice is maintained in a receptor/G protein reconstitution assay by using functional bovine brain G protein. These findings suggest that endocannabinoids acting at CB1 contribute to ethanol preference, and decreased coupling of CB1 to G proteins in the limbic forebrain by mechanisms other than altered receptor or G protein levels may be involved in the age-dependent decline in the appetite for both ethanol and food.

Alcohol is known to differentially affect neurotransmission in the brain, and pharmacological modulation of certain neurotransmitter systems has been found to reduce alcohol preference in rodents and alcohol consumption by alcoholics. Neurotransmitters implicated in controlling ethanol (EtOH) preference, such as norepinephrine (1), dopamine (2), serotonin (3, 4), endogenous opioids (5), and neuropeptide Y (6), have been also found to play important roles in the control of appetite and food intake (7), lending credence to the notion that alcoholism is an appetitive disorder.

Endogenous cannabinoids are recently discovered lipid mediators (8) that produce their effects via interaction with specific cannabinoid receptors (CBs) such as the brain type, or CB1 (9), and CB2 expressed predominantly in cells of the immune system (10). Endogenous cannabinoids have been implicated in a growing number of biological functions, including the control of appetite and food intake. We recently reported that food-restrained mice deficient in CB1 ($CB1^{-/-}$ mice) eat less than their wild-type littermates ($CB1^{+/+}$ mice), and the selective CB1 antagonist SR141716A reduces food intake in $CB1^{+/+}$ but not in $CB1^{-/-}$ mice (11). These findings indicate that endogenous cannabinoids are positive modulators of food intake, providing a rational explanation of the well known appetite stimulating effect of marijuana. SR141716A has also been reported to reduce voluntary EtOH intake in rodent models of EtOH drinking behavior (12–15). Although this could suggest a role for endogenous cannabinoids in the control of EtOH preference, the well known inverse agonist property of SR141716A (16) makes such a conclusion tenuous. In the present study, we examined the possible role of endogenous cannabinoids in EtOH preference using $CB1^{+/+}$ and $CB1^{-/-}$ mice developed on a C57BL/6J background (17), a strain known to have high preference for EtOH (18). The results indicate that endogenous cannabinoids acting at CB1 can be implicated in the high EtOH preference of young C57BL/6J mice, and reduced CB1 signaling in the limbic forebrain may be involved in the age-dependent decline in both EtOH preference and food intake.

Materials and Methods

Animals. $CB1^{-/-}$ and $CB1^{+/+}$ mice have been developed as described (17). Briefly, the CB1 gene was mutated in MPI2 embryonic stem cells, and chimeric and heterozygous animals were backcrossed to C57BL/6J mice. $CB1^{-/-}$ and $CB1^{+/+}$ littermates were obtained by interbreeding heterozygous mice, and genotype was established by a PCR-based assay using DNA extracted from the tail. All animals were housed under a 12-h light/dark cycle and had access to food and water ad libitum, except when stated otherwise. Animals used were defined as either young (6–10 weeks of age) or old (26–48 weeks of age) at the time of the experiment.

Alcohol Intake and Preference. To quantify EtOH preference, a two-bottle free-choice paradigm was used. Alcohol-naive mice were individually housed in cages with access to two identical bottles containing water vs. 10% or 20% EtOH in water. Animals were allowed to adapt to the paradigm for 7 days before the test day. The ratio of the 24-h intake from the EtOH bottle vs. total fluid intake was used to define preference, and the absolute amount of alcohol consumed was also calculated.

Blood Alcohol Concentration. Fresh bottles containing measured amounts of drinking water and alcohol solution were placed in cages at the onset of the dark period. Two hours later, the amount of liquids consumed was measured, and the animals were killed by decapitation. Plasma levels of alcohol were determined from trunk blood by an enzymatic method using the Sigma diagnostic alcohol reagent.

Food Intake. Cumulative food intake in 18-h food-restricted mice was quantified during the first 3 h of the dark period by measuring the amount of mouse chow left on a tray at 0, 1, 2, and 3 h and correcting for spillage, which was minimal.

Endocannabinoid Levels in Brain Tissue. Mice were decapitated and the brain quickly (<30 s) removed. After removal of the cerebellum, the forebrain was removed by a coronal cut across the optic chiasm. After removal of the olfactory bulb, the

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Abbreviations: CB, cannabinoid receptor; 2-AG, 2-arachidonoylglycerol; EtOH, ethanol; GTP[γS], guanosine 5′-[γ-thio]triphosphate.

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remaining forebrain tissue, which contained the nucleus accumbens and the anterior cingulate cortex, was referred to as limbic forebrain. The hypothalamus was then isolated by a second coronal cut through the mamillary bodies, two parallel sagittal cuts at the lateral edge of the optic tracts, and a horizontal cut across the top of the third ventricle. Finally, the lower parietal regions containing the pyriform cortex and the amygdala were dissected free. The four samples were frozen on dry ice and weighed. Brain tissue samples from individual mice, weighing 30–60 mg, were homogenized in 0.5 ml of an ice-cold solution of methanol/Tris buffer (50 mM, pH 8.0), 1:1, containing 7 ng of d4-anandamide, synthesized as described (19). To each homogenate, 2 ml of ice-cold chloroform/methanol (1:1) and 0.5 ml of 50 mM Tris buffer, pH 8.0, was added. The homogenate was centrifuged at 4°C (500 × *g* for 2 min), the chloroform phase was recovered and transferred to a borosilicate tube, and the water phase was extracted two more times with ice-cold chloroform. The combined extract was evaporated to dryness at 32°C under a stream of nitrogen. The dried residue was reconstituted in 110 μl of chloroform, and 2 ml of ice-cold acetone was added. The precipitated proteins were removed by centrifugation (1,800 × *g*, 10 min), and the clear supernatant was removed and evaporated to dryness. The dry residues were reconstituted in 50 μl of ice-cold methanol, of which 35 μl was used for analysis by liquid chromatography/in line mass spectrometry, by using an Agilent 1100 series LC-MSD, equipped with a thermostated autosampler and column compartment. Liquid chromatographic separation of endocannabinoids was achieved by using a guard column (Discovery HS C18, 2 cm × 4.0 mm, 3 μm, 120A) and analytical column (Discovery HS C18, 7.5 cm × 4.6 mm, 3 μm) at 32°C with a mobile phase of methanol/water/acetic acid (85:15:0.1, vol/vol/vol) at a flow of 1 ml/min for 12 min followed by 8 min of methanol/acetic acid (100:0.1, vol/vol). The MSD (model LS) was set for atmospheric pressure chemical ionization, positive polarity, and selected ion monitoring to monitor ions *m/z* 348 for AEA, 352 for d4-AEA, and 379 for 2-arachidonoylglycerol (2-AG). The spray chamber settings were as follows: vaporizer, 400°C; gas temperature, 350°C; drying gas, 5.0 liters/min; and nitrogen was used as the nebulizing gas with a pressure of 60 psig. Calibration curves were produced by using synthetic anandamide and 2-AG (Cayman Chemical, Ann Arbor, MI). The amounts of AEA and 2-AG in the samples were determined by using inverse linear regression of standard curves. Values are expressed as fmol or pmol per mg wet tissue.

Radioligand Binding. The density of CB1 was assessed as described (20). Briefly, the cerebellum, limbic forebrain, amygdala, and hypothalamus were dissected from young and old $CB1^{+/+}$ mice. The tissue was homogenized in 5 ml of ice-cold buffer A (320 mM sucrose/50 mM Tris·HCl/2 mM EDTA/5 mM $MgCl_2$, pH 7.4). The homogenate was centrifuged at 1,000 × *g* for 10 min at 4°C. The supernatant was saved, and the pellet was resuspended in fresh buffer A and washed twice more with subsequent centrifugations. The combined supernatants were centrifuged at 40,000 × *g* for 30 min. The pellet was resuspended in 2 ml of buffer B (50 mM Tris·HCl/1 mM EDTA/3 mM $MgCl_2$, pH 7.4) to yield a protein concentration of 1 mg/ml. Binding was initiated by the addition of 50 μg of membrane protein to silanized tubes containing a saturating concentration (2 nM) of [^{3}H]SR141716A (52.0 Ci/mmol, Amersham Pharmacia) and buffer C (buffer B supplemented with 5 mg/ml BSA) to bring the total volume to 1 ml. Nonspecific binding was established in the presence of 1 μM unlabeled SR141716A. Triplicate aliquots were incubated at 30°C for 1 h, followed by the addition of 2 ml of ice-cold buffer D (50 mM Tris·HCl/1 mg/ml BSA, pH 7.4) and vacuum filtration through GF/B filters presoaked in buffer D. Retained radioactivity was quantified by liquid scintillation spectrometry. The use of a single saturating concentration of the radioligand instead of full saturation isotherm was necessitated due to the small size of tissue and the limited number of homozygous animals available. In pilot experiments, the K_d of [^{3}H]SR141716A binding determined from saturation isotherms was 0.15–0.2 nM using membranes prepared from whole mouse brain, in agreement with a published report (21).

Guanosine 5′-[γ-thio]Triphosphate (GTP[γS]) Binding. Agonist-stimulated [^{35}S]GTP[γS] binding was measured as described (21). Briefly, 50 μg of membrane protein was incubated in 0.5 ml of TME-Na buffer (50 mM Tris·HCl/0.2 mM EGTA/3 mM $MgCl_2$/100 mM NaCl, pH 7.4) containing 0.1 mg/ml BSA, 30 μM GDP, 0.1 nM [^{35}S]GTP[γS] (1250 Ci/mmol, NEN Life Science), with or without HU-210 in siliconized glass tubes. Triplicate aliquots were incubated at 30°C for 1 h, and the reaction was terminated by the addition of 2 ml of ice-cold wash buffer (50 mM Tris·HCl, pH 7.4), followed by vacuum filtration through GF/B filters, and the retained radioactivity was quantified by liquid scintillation spectrometry.

Membrane/G Protein Reconstitution Assay. These assays were performed as described (22). Briefly, brain tissue is homogenized in 5 ml of ice-cold lysis buffer (5 mM Tris·HCl, pH 7.5/5 mM EGTA/5 mM EDTA/0.1 mM PMSF/1 μg/ml pepstatin/1 μg/ml aprotinin). The lysate is centrifuged at 17,000 × *g* for 15 min, and the pellet is resuspended in 0.5 ml of buffer A (50 mM Tris·HCl, pH 7.4/0.6 mM EDTA/5 mM $MgCl_2$) and incubated with 100 ng/ml pertussis toxin at 4°C for 2 h. Membranes are solubilized with 1 ml of 1% sodium cholate for 30 min at 4°C. The material is centrifuged at 100,000 × *g* for 1 h, and the supernatant is used for the assay. A single assay point consisted of six tubes, two for total binding, two for nonspecific binding defined in the presence of 100 μM GTP[γS], and two for the agonist addition (100 nM HU-210). A 5× preincubation mixture prepared for each tube contained 3 μg of functional bovine brain G protein (Calbiochem) and 60 μg of membrane protein in 120 μl of buffer A and 50 μM GDP. After 1 h at 4°C, 20 μl of the preincubation mixture is added to the tubes containing 80 μl of buffer A plus (final concentrations) 150 mM NaCl, 1 mM DTT, 0.2 nM [^{35}S]GTP[γS], and either vehicle, 100 nM GTP[γS] or 100 nM HU-210. Incubation at 24°C for 30 min is terminated by vacuum filtration as described above.

Western Blotting. Tissue levels of G protein subunits or RGS proteins were quantified by Western immunoblotting as described (20). All antibodies were purchased from Santa Cruz Biotechnology. The T-19 antibody is specific for $G_i\alpha_2$ and non-crossreactive with $G_i\alpha_1$ or $G_i\alpha_3$; the C-10 antibody is reactive with $G_i\alpha_1$, $G_i\alpha_2$, and $G_i\alpha_3$ and non-crossreactive with other Giα subunit proteins. The K-20 antibody is specific for $G_o\alpha$, and the T-20 antibody is broadly reactive with $G\beta_1$, $G\beta_2$, $G\beta_3$, and $G\beta_4$. N-16 and C-20 are specific antibodies for RGS4 and RGS10, respectively.

Drugs. SR141716A (*N*-[piperidin-1-yl]-5-[4-chlorophenyl]-1-[2,4-dichlorophenyl]-4-methyl-1H-pyrazole-3-carboxamide HCl) was synthesized and provided by the National Institute on Drug Abuse; anandamide (arachidonyl ethanolamide) was from Sigma. HU-210 (Δ^9-tetrahydrocannabinol dimethylheptyl) was synthesized and kindly provided by Dr. Raphael Mechoulam. SR141716A, anandamide, and HU-210 were dissolved in alkmulphor/EtOH/saline (1:1:18) and injected i.p. in volumes of 5–10 μl. Alkmulphor is a vegetable oil (Rhodia, Cranbury, NJ).

Statistical Analyses. Comparison of the means from multiple treatment groups was done by using ANOVA followed by Duncan's post hoc test. For comparing pre- and posttreatment

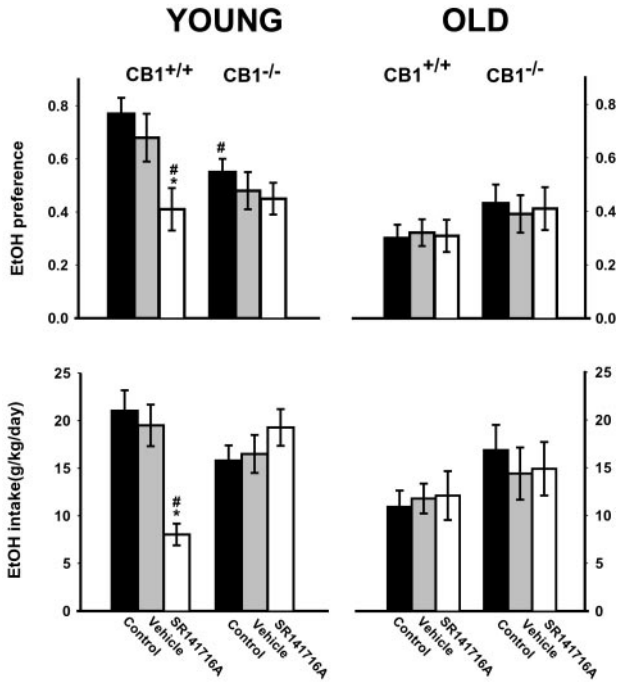

Fig. 1. EtOH preference and intake are reduced by pretreatment with SR141716A in young CB1$^{+/+}$ mice, but not in old CB1$^{+/+}$ mice or in young and old CB1$^{-/-}$ mice. Mice aged 26–48 weeks were tested in a two-bottle (water vs. 20% EtOH) free-choice paradigm as described in *Materials and Methods*. The animals were untreated (control) or pretreated i.p. with vehicle or 3 μg/g SR141716A 30 min before the start of the testing period. Columns and bars represent mean ± SE from 11 young CB1$^{+/+}$, 18 young CB1$^{-/-}$, 10 old CB1$^{+/+}$, and 14 old CB1$^{-/-}$ mice. Significant difference ($P < 0.05$) from corresponding value in untreated (#) or vehicle-treated CB1$^{+/+}$ mice (*) is indicated. Total fluid intake (3.5–6 ml per mouse per day for young and 5–8 ml per mouse per day for old mice) was unaffected by either vehicle or SR141716A treatment.

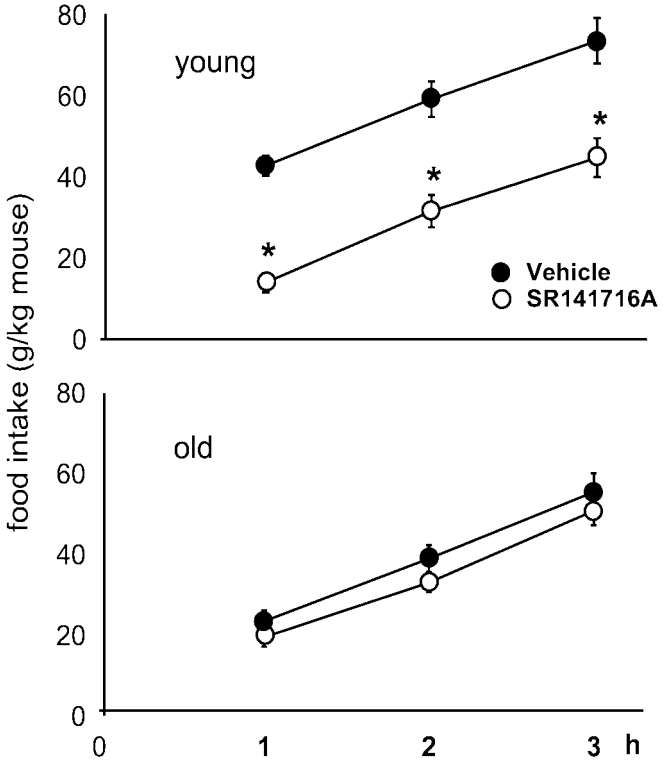

Fig. 2. Food intake is reduced by SR141716A in young but not in old CB1$^{+/+}$ mice. Food intake was tested after an 18-h food restriction in young (*Upper*) and old (*Lower*) CB1$^{+/+}$ mice, pretreated with vehicle (filled symbols) or 3 μg/g SR141716A (open symbols) as described in *Materials and Methods*. Significance of difference ($P < 0.05$) between vehicle- and SR141716A-treated young mice (*) is indicated. Eight to 10 animals were tested in each group.

values in the same animals, the paired *t* test was used. Values with a $P < 0.05$ were considered statistically significant.

Results

EtOH Preference Is Higher in Young Than in Old CB1$^{+/+}$ or Young CB1$^{-/-}$ Mice. EtOH preference and intake were determined in a two-bottle, free-choice paradigm by using a 20% or 10% EtOH solution in water vs. water. Due to an interruption in our breeding program, only old animals were available for the first series of experiments. By using the 20% EtOH vs. water paradigm, EtOH preference and intake were similar in old CB1$^{+/+}$ mice and their CB1$^{-/-}$ littermates and were unaffected by pretreatment of mice with vehicle or 3 μg/g SR141716A in either group (Fig. 1 *Right*).

When new litters became available, similar experiments were done by using animals 6–10 weeks of age. EtOH preference in the young CB1$^{+/+}$ mice (Fig. 1 *Left*) was higher than in the old mice, and both the high preference (*Upper*) and the absolute amount of EtOH consumed (*Lower*) were significantly reduced by SR141716A, which did not affect total fluid intake. The difference was significant compared either to untreated control or vehicle-treated mice in the same group. In contrast, in CB1$^{-/-}$ mice, EtOH preference and intake were similar in young and old animals and were unaffected by SR141716A pretreatment (Fig. 1). Similar results were obtained by using the 10% EtOH vs. water paradigm; in CB1$^{+/+}$ mice, EtOH intake (g/kg mouse) was reduced by SR141716A in young (13.3 ± 1.9 vs. 5.1 ± 1.7, vehicle vs. SR, $P < 0.01$) but not in old animals (8.2 ± 1.7 vs. 6.2 ± 1.0), whereas in CB1$^{-/-}$ mice, EtOH intake was unaffected by SR141716A in both young (9.9 ± 2.1 vs. 10.7 ± 2.3, vehicle vs. SR) and old (8.1 ± 1.7 vs. 8.0 ± 0.9) mice.

Serum EtOH Levels in Free-Drinking Mice. To test whether mice drink sufficient alcohol to produce psychotropic effects, serum alcohol levels were measured in six young CB1$^{+/+}$ mice with free access to 20% EtOH or water. Because mice drink during the dark phase, the animals were killed 2 h after the beginning of the dark phase. Serum alcohol levels ranged from 3.0 to 137.0 mg/dl and were highly correlated ($r = 0.86$) with the amount of EtOH consumed during the preceding 2-h period (0.450–6.2 g/kg). Three of the six mice had serum EtOH levels >100 mg/dl (>22 mM).

Food Intake Declines with Age in CB1$^{+/+}$ Mice. The results of a recent study indicate that endocannabinoids acting at CB1 are involved in the control of food intake in C57BL/6J mice (11). Because food intake is known to decline with age (23), we tested whether this may also be related to the loss of CB1 involvement. As illustrated in Fig. 2, food intake in food-restricted mice appeared higher in young than in old CB1$^{+/+}$ mice, and it was inhibited by SR141716A only in the young animals.

Brain Endocannabinoid Levels Are Similar in Old and Young Mice. To test whether age-dependent differences in endocannabinoid levels may account for or accompany the disappearance of SR141716A-sensitive EtOH intake in older CB1$^{+/+}$ mice, tissue levels of anandamide and 2-AG were analyzed in four brain regions of young and old CB1$^{+/+}$ mice. No significant age-dependent differences were found in any of the regions studied for either of the two endocannabinoids. Anandamide levels

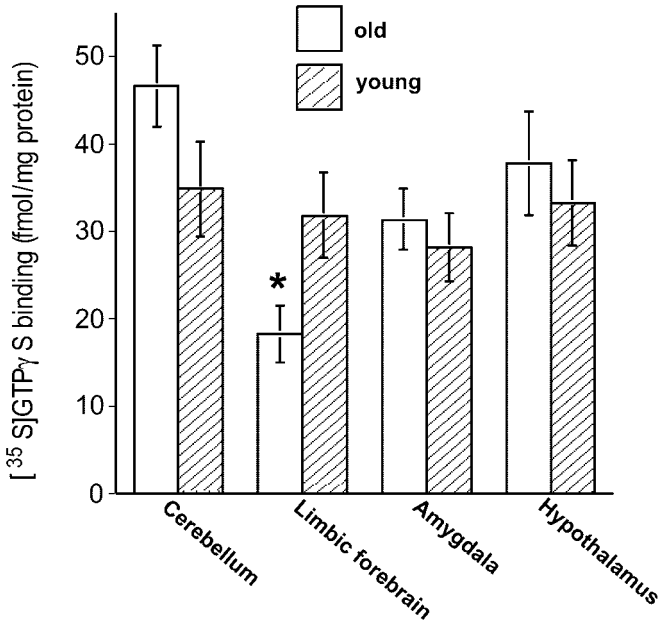

Fig. 3. Cannabinoid agonist-stimulated [^{35}S]GTP[γS] binding is selectively reduced in the limbic forebrain of old compared with young CB1$^{+/+}$ mice. [^{35}S]GTP[γS] binding was measured in membrane preparations from cerebellum, hypothalamus, amygdala, and limbic forebrain from 13 old (open columns) and 16 young (hatched columns) CB1$^{+/+}$ mice as described in *Materials and Methods*. Columns and bars represent means ± SE of the difference in GTP[γS] binding measured in the absence and presence of 100 nM HU-210.

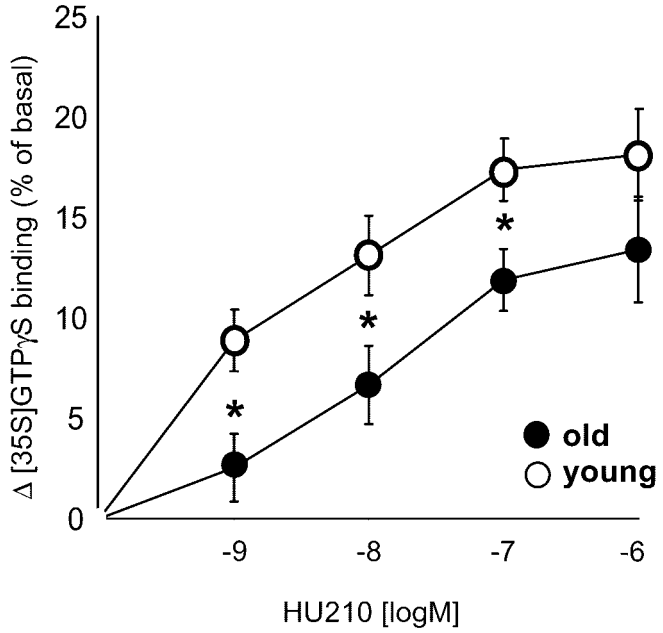

Fig. 4. Concentration dependence of HU-210-stimulated GTP[γS] binding in membrane preparations from the limbic forebrain of old (●) and young (○) CB1$^{+/+}$ mice. Points and vertical bars represent means ± SE (n = 9 in each group) of the percentage increase GTP[γS] binding in the presence of different concentrations of HU-210 over baseline binding in the absence of the agonist. *, significant difference ($P < 0.05$) between corresponding points in old and young mice.

(fmol/mg tissue, n = 8) in young vs. old rats were 5.3 ± 0.6 vs. 5.5 ± 0.7 in cerebellum, 3.2 ± 1.4 vs. 3.2 ± 1.3 in limbic forebrain, 8.6 ± 0.8 vs. 8.9 ± 1.2 in amygdala, and 9.6 ± 1.6 vs. 8.6 ± 0.3 in hypothalamus. 2-AG levels in the same samples were (pmol/mg tissue, n = 8) 9.2 ± 0.7 vs. 8.0 ± 0.4, 9.3 ± 1.8 vs. 9.1 ± 1.2, 14.0 ± 2.2 vs. 10.8 ± 0.6, and 9.7 ± 0.3 vs. 9.2 ± 1.7.

CB1 Density Is Similar in Old and Young Mice. We next tested whether a decline in the expression of CB1 may account for the loss of SR141716A-reducible EtOH preference in older CB1$^{+/+}$ mice by measuring the specific binding of a saturating concentration of the CB1-specific radioligand, [^{3}H]SR141716A, to membranes prepared from different brain regions. Again, there were no age-dependent, statistically significant differences in CB1 density in any of the brain regions studied. Receptor densities (pmol/mg protein, mean ± SE) in nine old vs. nine young mice were 3.55 ± 0.45 vs. 3.48 ± 0.48 in cerebellum, 3.36 ± 0.50 vs. 3.84 ± 0.42 in limbic forebrain, 4.11 ± 0.57 vs. 4.61 ± 0.58 in amygdala, and 2.67 ± 0.37 vs. 2.87 ± 0.37 in hypothalamus.

CB1 Coupling Is Selectively Reduced in the Limbic Forebrain of Old Mice. In the absence of changes in the cellular level of CB1, changes in the efficiency of their coupling to the G protein-linked signaling cascade may account for changes in endocannabinoid signaling. CB1 are coupled to the G_i protein, and their activation leads to dissociation of GDP and the subsequent binding of GTP to $G_i\alpha$. The agonist-stimulated binding of the nonhydrolyzable GTP analog GTP[γS] can be used as an indicator of agonist efficacy or the efficiency of receptor coupling. We therefore measured [^{35}S]GTP[γS] binding in the absence and presence of a maximally effective concentration (100 nM) of the potent CB1 agonist HU-210 in brain membranes from young and old CB1$^{+/+}$ mice. As illustrated in Fig. 3, in young animals agonist-stimulated GTP[γS] binding was similar in the four brain regions studied. In old animals, the level of agonist-stimulated GTP[γS] binding was similar to that in young animals in the cerebellum, amygdala, and hypothalamus. However, there was a marked and statistically significant decrease in agonist-stimulated GTP[γS] binding in the limbic forebrain. This is illustrated further in Fig. 4 where the agonist concentration dependence of stimulated GTP[γS] binding in membranes from the limbic forebrain of young and old animals is shown.

To further explore the possible mechanism underlying the decreased coupling of CB1, the level of expression of G_i and G_o protein subunits was quantified by Western immunoblotting in membranes prepared from the limbic forebrain of young and old CB1$^{+/+}$ mice. By using antibodies against $G_i\alpha_{1-3}$, $G_i\alpha_2$, $G_i\beta_{1-4}$, and $G_o\alpha$ in preparations from four young and four old mice, no significant age-dependent differences were found, as quantified by densitometry (not shown). Next, we analyzed receptor coupling in a signal reconstitution assay where the receptor has access to the same population of G proteins. CB1 coupling to endogenous G_i/G_o was eliminated by pertussis toxin pretreatment of the membrane preparations, and signal transfer was restored by reconstitution of the cell membranes with functional bovine G protein. When HU-210-stimulated [^{35}S]GTP[γS] binding was compared in membranes from young and old CB1$^{+/+}$ mice by using this signal-restoration assay, similar levels of binding were detected in the cerebellum, whereas in the limbic forebrain binding was significantly lower in preparations from old compared with young mice (Fig. 5). We also quantified two of the more common RGS proteins, which are accessory factors operating at the receptor/G protein interface. Western blotting using antibodies against RGS4 and RGS10 revealed no significant difference in the level of these two proteins, as tested in membranes from the limbic forebrain of four young and four old CB1$^{+/+}$ mice (not shown).

Possible Link Between Reduced EtOH Preference and CB1 Signaling in Old Mice. To examine a possible cause and effect relationship between reduced EtOH preference and CB1 signaling in old

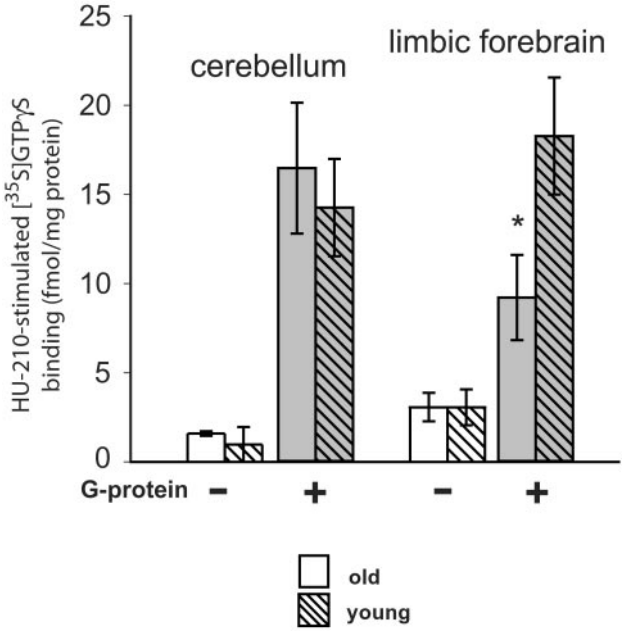

Fig. 5. HU-210-stimulated GTP[γS] binding in pertussis toxin-pretreated membranes reconstituted with bovine brain G protein. Membranes were prepared from cerebellum and limbic forebrain of young (hatched columns) and old (open columns) $CB1^{+/+}$ mice and incubated in the absence (−) or presence (+) of bovine brain G protein. Preparation and treatment of membranes and assay of [^{35}S]GTP[γS] binding were as described in *Materials and Methods*. *, significant difference ($P < 0.05$) from corresponding value in membranes from young animals.

mice, we tested whether treatment of old $CB1^{+/+}$ mice with a CB1 agonist can increase voluntary EtOH intake in the 10% EtOH vs. water paradigm. We first treated mice with 20 ng/g HU-210 (i.p.), which caused marked behavioral inhibition including hypomotility, associated with a drastic reduction in both food and total fluid intake (not shown). Because the behavioral effects of HU-210 are known to interfere with ingestive behavior (24), we next tested anandamide at a dose (1 μg/g s.c.) that is subthreshold for causing the cannabinoid-like tetrad effects including hypomotility (25), but is maximally effective in increasing food intake (26). Treatment of six old $CB1^{+/+}$ mice with anandamide did not influence their total fluid intake, but 24-h EtOH intake increased by 46 ± 13% ($P < 0.05$) from 8.7 to 12.6 g/kg body weight per day, whereas combined treatment of six other old $CB1^{+/+}$ mice with 3 μg/g SR141716A plus 1 μg/g anandamide did not affect 24-h EtOH intake (−17 ± 21%, from 8.0 to 6.9 g/kg per day).

Discussion

The present findings provide unequivocal evidence for the role of endocannabinoids and CB1 in alcohol-drinking behavior in rodents and suggest that the age-dependent decline in EtOH preference may be linked to a parallel decline in CB1 signaling. They further suggest that the same mechanism may be involved in the age-dependent decline in food intake.

The significantly higher EtOH preference and intake of young wild-type mice compared with their $CB1^{-/-}$ littermates, and the ability of SR141716A to reduce EtOH drinking in wild-type but not in $CB1^{-/-}$ mice, clearly indicates that activation of CB1 contributes to the high preference for EtOH of C57BL/6J mice. These findings are in agreement with recent reports that SR141716A can reduce EtOH intake in rodent models of alcohol drinking (see Introduction) and indicate that the effect of SR141716A is due to CB1 antagonism rather than inverse agonist activity or a nonspecific effect. They are also in agreement with the reported ability of CP-55,940, a highly potent CB1 agonist, to increase the motivation of rats to drink alcohol (27). The inability of SR141716A to inhibit alcohol drinking in older wild-type or in $CB1^{-/-}$ mice is unlikely to be due to a floor effect, as these animals still consumed significant amounts of alcohol. Rather, this finding suggests that endocannabinoids are involved in the drive to drink only over a certain threshold. This is similar to their involvement in the hunger-induced increase in food intake, but not in basal levels of food ingestion in sated animals (11).

CB1 are the most abundant neurotransmitter receptors in the brain. Although they are also present in some peripheral tissues, CB1-mediated behavioral effects, such as the modification of ingestive behaviors, are believed to be triggered in the brain. Interestingly, in young wild-type mice, SR141716A reduced EtOH preference and intake to levels below those seen in knockout mice (see Fig. 1). This could suggest that compensatory changes in other neurotransmitter systems in knockouts may have offset some of the effects of lack of cannabinoid signaling.

Although unexpected, the observed age-dependent decline in EtOH preference in wild-type mice parallels observations in humans, in that only some of the teenage binge drinkers become alcoholics as adults, and that the onset of alcoholism declines with age (28). Interestingly, a study published almost 40 years ago demonstrated that in BALB/c mice, which are known to avoid alcohol, alcohol preference measured in a paradigm similar to that used in the present study was as high as 40% in the first 10 weeks of life, after which preference declined to around 10% (29). Thus, the age dependence of EtOH preference manifests not only longitudinally in a given strain but also across strains with genetically based differences in EtOH preference.

Unlike wild-type mice, young $CB1^{-/-}$ mice displayed low EtOH preference, which remained unchanged as the animals got older. Furthermore, the age-dependent decline in EtOH preference in wild-type mice was associated with a loss of the ability of SR141716A pretreatment to further reduce EtOH preference and intake. These findings strongly suggest that the decline in EtOH preference is related to a loss of cannabinoid signaling in a relevant brain area. As a further indication for a cause and effect relationship between these two phenomena, treatment of old wild-type mice with a low dose of anandamide was able to cause a small but significant increase in voluntary EtOH intake, and this effect could be prevented by simultaneous treatment with the CB1 antagonist SR141716A.

Age-dependent differences in the brain levels of endocannabinoids and their precursors (30), as well as of CB1 (31) and CB1-mediated effects on synaptic transmission (32), have been reported. However, in $CB1^{+/+}$ mice, no age-dependent changes in anandamide and 2-AG levels or CB1 densities could be detected in four brain regions. Three of these, the hypothalamus, the limbic forebrain that includes the nucleus accumbens, and the nucleus amygdala comprise the most important structures involved in the central neural control of appetite and food intake. Although changes limited to a smaller subregion may have gone undetected, these findings suggest that a decrease in endogenous mediators or in the cellular density of their receptors is unlikely to account for the loss of cannabinoid-mediated EtOH preference in old mice.

In the absence of measurable changes in ligand or receptor levels, the observed striking, selective reduction in agonist-stimulated GTP[γS] labeling in the limbic forebrain of old mice could suggest that a localized decline in the coupling of CB1 to G proteins may account for the reduced alcohol preference. A variety of mechanisms may influence receptor/G protein coupling. These include, although are not limited to, changes in receptor trafficking, posttranslational covalent modification of G proteins (33) or receptors, and the presence of accessory

proteins such as RGS proteins (34) or receptor activity-modifying proteins (35). Although the molecular mechanism(s) underlying the region-specific change in CB1-stimulated GTP[γS] labeling remain to be determined, the present findings allow some conclusions. The level of G_o/G_i proteins in the brain is in excess of most G protein-coupled receptors (36). However, CB1 is expressed in the brain at such a high level that a reduction in the expression of G_o/G_i protein subunits may become limiting for CB1 signaling in some brain regions. However, this is unlikely in view of the unchanged levels of G_i and G_o subunit proteins in young and old mouse brain, and also of the finding that the selective reduction in GTP[γS] labeling in the limbic forebrain of old mice is retained in a receptor/G protein reconstitution assay. Alternatively, covalent modification of CB1 such as protein kinase C-induced phosphorylation, which has been shown to impair CB1 signaling (37), could result in reduced GTP[γS] labeling.

A part of the limbic forebrain, the nucleus accumbens, is a major site for mediating the rewarding properties of both alcohol (38) and cannabinoids (39, 40), and it has also been implicated in the regulation of appetite (41). These effects are thought to be mediated through increases in dopamine release in the nucleus accumbens (42), which has been documented both for cannabinoids (43) and EtOH (44). Furthermore, a selective decrease in glutamate-induced dopamine release in the accumbens but not in the striatum of aged rats has been reported (45). Thus, this region of the brain is a plausible site for the age-dependent interaction of EtOH and endocannabinoids.

The well documented anorexia of old age (23) is analogous to the decline in food intake we observed in old $CB1^{+/+}$ mice, which also became insensitive to inhibition by SR141716A (Fig. 2). This suggests that the nucleus accumbens or another structure in the limbic forebrain is a likely site for the parallel, age-dependent decline in food as well as EtOH intake. Of interest, the results of a recent study indicate that GTP[γS] binding stimulated by the cannabinoid agonist CP-55,940 was markedly lower in brain membranes prepared from alcohol avoiding DBA/2 mice than in preparations from the alcohol preferring C57BL/6J mice (46). These and other observations, indicating that chronic exposure of mice to EtOH down-regulates CB1-stimulated GTP[γS] binding (47), further suggest that CB1 signaling is both a neuronal target of EtOH and a determinant of EtOH preference. The molecular mechanism(s) of the region-specific change in GTP[γS] labeling and its possible behavioral consequences remain to be determined.

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